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Application of rRNA-Based Probes for Observing Marine Nanoplanktonic Protists†

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The use of small-subunit rRNA-based oligonucleotides as probes for detecting marine nanoplanktonic protists was examined with a ciliate (an *Uronema* sp.), a flagellate (a *Cafeteria* sp.), and mixed assemblages of protists from enrichment cultures and natural seawater samples. Flow cytometry and epifluorescence microscopy analyses demonstrated that hybridizations employing fluorescein-labeled, eukaryote-specific probes intensely stained logarithmically growing protists, whereas these same protist strains in late stationary growth were barely detectable. The fluorescence intensity due to probe binding was significantly enhanced by the use of probes end labeled with biotin, which were detected by fluorescein-labeled avidin. The degree of signal amplification ranged from two- to fivefold for cultured protists in both logarithmic and stationary growth phases. Mixed assemblages of heterotrophic protists from enrichment cultures were also intensely labeled by rRNA-targeted oligonucleotide probes by the biotin-avidin detection system. Protists in late stationary growth phase and natural assemblages of protists that were otherwise undetectable when hybridized with fluorescein-labeled probes were easily visualized by this approach. In the latter samples, hybridization with multiple, biotin-labeled probes was necessary for detection of naturally occurring marine protists by epifluorescence microscopy. The signal amplification obtained with the biotin-avidin system should increase the utility of rRNA-targeted probes for identifying protists and facilitate characterization of the population structure and distribution of protists in aquatic environments.

Nanoplanktonic protists (2 to 20 μm in diameter) are recognized as fundamental components of aquatic ecosystems because of their multiple ecological roles (5). Within planktonic food webs, these microorganisms function as primary producers, nutrient remineralizers, and intermediaries in the transfer of energy to higher trophic levels. Photosynthetic and heterotrophic species within this assemblage are typically present at similar abundances (10^2 to 10^3 ml^{-1}) in plankton communities (7). Phototrophic protists often dominate total primary production (24), while heterotrophic protists typically are the primary consumers of bacteria, cyanobacteria, and microalgae (5, 10, 28, 29). Because of their high metabolic activity and their ability to ingest significant amounts of particulate organic material, heterotrophic protists have also been implicated as major nutrient remineralizers in the marine environment (5, 8, 9).

Although the ecology of the nanoplankton has been fairly well characterized, our knowledge of the population structure and species composition of this assemblage remains very limited. The biogeography of most nanoplanktonic species has been poorly studied, and thus the spatiotemporal distributions of many species are virtually unknown. These shortcomings stem primarily from the difficulties associated with identifying protists in natural water samples. Nanoplankton are typically enumerated by direct counting procedures using epifluorescence microscopy and fluorochrome staining (7, 18, 27). With this method, phototrophic (chloroplast-bearing) species are distinguished from nonpigmented protists by the autofluorescence of chlorophyll *a* (7, 12). Other than this crude separation, however, epifluorescence microscopy cannot provide enough detail for the identification of even the broad taxonomic affinities of most small protists. Sufficient criteria for taxonomic characterization of these microorganisms must still be obtained by electron microscopy, but this method is time-consuming, expensive, and impractical for analyses of large numbers of samples. Other cytological techniques for taxonomic studies are technically complicated and not feasible for routine examination of samples.

An alternative to conventional methods for identifying protistan taxa is the use of nucleic acid hybridization probes. Nucleic acid probes have long been used to detect specific DNA or RNA sequences in tissue sections or intact cells (6, 11, 16, 22). Small-subunit rRNA-based oligonucleotide probes in particular are becoming an increasingly useful tool for characterizing microbial cells in cultures as well as in clinical or environmental samples (1, 3, 13, 15, 17, 32). The ubiquity, intermediate size, and variable sequence conservation of the small-subunit rRNA facilitate the design of probes capable of distinguishing various phylogenetic groupings, ranging from domains (17) to species (2, 14, 31). Because these probes are short oligonucleotides (18 to 20 bp long), formalin-fixed cells are relatively permeable to the probes. Moreover, the relatively high cellular content of rRNA provides reasonably abundant target sites for probe binding.

The use of rRNA probes to discriminate numerous bacterial species is now common (3, 19, 21, 23, 33), but rRNA probes have not yet been designed for planktonic protists. Protists are potentially more amenable to detection with oligonucleotide probes than bacteria because protists are larger than bacteria and thus more likely to contain more ribosomes and higher concentrations of rRNA. This fact is especially important in nonradioactive detection systems, which are generally less sensitive than radiolabeled probes.

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Furthermore, nonradioactive probes such as fluorescently labeled rRNA probes (14) are well suited for studies with protists, because methods for sample preparation and detection are adaptable to epifluorescence microscopy techniques commonly used to count protists in ecological studies.

This study was designed to test the suitability of eukaryote-specific, fluorescently labeled rRNA probes to label protists for detection and enumeration. Preliminary hybridization experiments demonstrated that fluor-labeled oligonucleotide probes intensely labeled ciliates and flagellates in the logarithmic growth phase. However, the fluorescence due to probe binding in stationary-growth-phase cells was relatively weak. These results stimulated us to investigate alternative labeling and detection methods to amplify the fluorescent signal, so that cells would be labeled regardless of their physiological state. Amplification of signal strength was achieved with biotin-labeled probes, whose binding was detected by subsequent treatment with fluorescein-labeled avidin. Cultured species of protists in late stationary growth phase, as well as natural assemblages of protists that were otherwise undetectable when hybridized with fluorescein-labeled probes, were readily visualized with this biotin-avidin detection system.

MATERIALS AND METHODS

Oligonucleotide synthesis and labeling. In situ hybridizations were performed with oligonucleotide probes complementary to discrete regions of eukaryotic small-subunit rRNA. The following eukaryote-targeted probes were used (the numbers correspond to the corresponding *Escherichia coli* sequence positions): EUK 1209R (5′-GGG CAT CAC AGA CCT G-3′) (17), EUK 502R (5′-ACC AGA CTT GCC CTC C-3′) (2), EUK 309R (5′-TCA GGC TCC CTC TCC GG-3′) (30), and EUK B (5′-TGA TCC TTC TGC AGG TTC ACC TAC-3′) (25). An eubacterial probe, EUB 338 (2), and a negative-control probe that binds the coding strand of the small-subunit rRNA gene (17) served as controls for nonspecific binding to eukaryotic cells.

The oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer. An amino group was attached to the 5′ end of the oligonucleotide in the last stage of synthesis (Aminolink II; Applied Biosystems). The 5′-aminohexyl oligonucleotides were labeled with either fluorescein or biotin as previously described (14), with some modifications. The labeling reaction mixture (250 μl) contained 100 μg of 5′-aminohexyl oligonucleotide, 100 mM carbonate buffer (pH 9.2), and 40 μl of a stock solution of 10 mg of fluorescein isothiocyanate (FITC) (Molecular Probes, Inc., Eugene, Oreg.) ml^{-1} or 33 μl of 100 mg of biotin-XX succinimidyl ester (Molecular Probes, Inc.) ml^{-1}. Reaction mixtures were incubated at room temperature for 16 h in the dark. Oligonucleotides were separated from unincorporated dye, or biotin, by passing the reaction mixture through a Sephadex G-25 column equilibrated with 10 mM Tris-HCl (pH 8.0). Eluted fractions containing the labeled oligonucleotides were subsequently lyophilized and resuspended with sterile double-distilled H_2O to a final volume of 50 μl. Separation of labeled oligonucleotides from unlabeled oligonucleotides was accomplished by electrophoresis on a 20% nondenaturing polyacrylamide gel. The band of labeled oligonucleotides was visualized by UV fluorescence and excised from the gel. Oligonucleotides were eluted in 1-ml portions of sterile double-distilled H_2O on a rotary shaker and filtered through 0.2-μm-pore-size Acrodisc filters (Gelman Sciences). The labeled oligonucleotides were lyophilized and resuspended in sterile double-distilled H_2O to a final concentration of 50 ng μl^{-1}.

Hybridization with fluorescein-labeled probes. The suitability of fluorescently labeled eukaryote probes for detecting protists was evaluated by flow cytometry and epifluorescence microscopy. Flow cytometric analyses were performed on a ciliate, an *Uronema* sp., sampled over its growth cycle and hybridized with a eukaryote-specific probe (EUK 1209R) (17). Ciliates were grown in sterile seawater enriched with 0.05% yeast extract and inoculated with bacteria. Cultures were sampled every 6 h over a 48-h period and every 24 h thereafter, until the culture was well into the stationary phase of growth. Samples were fixed in formaldehyde to a final concentration of 3.7% and stored at 4°C. Cell counts were performed on subsamples using a Fuchs-Rosenthal or Reichert hemocytometer. The remainders of the samples (15 ml) were concentrated by centrifugation to approximately 1 ml and used for flow cytometric analyses.

Separate cultures of this ciliate and a flagellate, a *Cafeteria* sp., were also grown as described above for hybridization and examination by epifluorescence microscopy and photometry. Cells were harvested in the logarithmic phase of growth, and in the late stationary phase of growth, the cells were concentrated by centrifugation and fixed by resuspending the pellet in cold, fresh 3.7% formaldehyde. Cell suspensions were stored at 4°C and used for hybridizations within 24 h after fixation. The cells were hybridized with one probe (EUK 309R) or a combination of two probes (EUK 309R and EUK 502R) or three probes (EUK 309R, EUK 502R, and EUK 1209R). A probe complementary to the coding strand of the rRNA gene served as a negative control for nonspecific binding.

In situ hybridization of protists was performed as follows. Cell concentrates (10 μl) were spotted in the wells of gel-coated, Teflon-coated slides (Cel-Line Associates, Inc.) and allowed to air dry. After dehydration of the cell smears in a series of ethanol washes (50, 75, and 100% ethanol) (each wash 2 min), 10 μl of hybridization buffer (750 mM NaCl, 100 mM Tris-HCl [pH 7.8], 5 mM EDTA, 0.1% sodium dodecyl sulfate) was added to each cell smear, followed by the addition of the oligonucleotide probe to a final concentration of 5 ng μl^{-1}. The slides were placed in air-tight chambers containing a piece of buffer-saturated tissue paper and incubated at 40°C for 3 h. The slides were then washed in a solution containing 30 mM NaCl, 4 mM Tris-HCl (pH 7.8), and 0.2 mM EDTA at 45°C for 10 min, air dried, and mounted with Citifluor (Citifluor, Ltd., London, England). The cells were observed with a Zeiss Axioskop 20 epifluorescence microscope fitted with Omega (Brattleboro, Vt.) optical filters and various dyes, such as 4′,6-diamidino-2-phenylindole (DAPI) (excitation, U340; emission, GG 420; dichroic beamsplitter, 400 DCLP at 45°), fluorescein (excitation, 470 DF 40; emission, 520 EF LP; dichroic beamsplitter, 505 DR LP at 45°), and Texas red (excitation, 560 DF 40; emission, 635 DF 60; dichroic beamsplitter, 595 DR LP at 45°). Phase-contrast and epifluorescence micrographs of the samples were taken with a Zeiss MC 100 camera and Ektachrome 200 ASA color film.

Modifications to the hybridization procedure were made for flow cytometric analysis as follows. Approximately 300 μl of cell concentrate was pelleted in a microcentrifuge tube and resuspended in absolute ethanol. The cells were pelleted again and mixed with 20 μl of hybridization buffer and then with 5 μl of probe (EUK 1209R). The hybridization mixture was gently vortexed and incubated in a 40°C water bath. After 12 h, the hybridization mixture was brought to a

volume of 300 μl with phosphate-buffered saline (pH 7.4) and immediately analyzed.

Hybridization with biotin-labeled probes. In situ hybridization of fixed *Uronema* and *Cafeteria* cells was repeated with both fluorescein- and biotin-labeled probes and analyzed by a combination of epifluorescence microscopy and photometry. The following additions were made to the procedure using biotin-labeled probes. After hybridization of the cells, the slides were air dried and 10 μl of fluorescein-labeled avidin solution (20 μg ml^{-1} in 100 mM carbonate-buffered saline [pH 8.2]) was added to each cell smear. The slides were incubated in the dark at 4°C for 10 to 20 min and subsequently washed three times with cold buffered saline. Cells incubated with only fluorescein-labeled avidin, but no oligonucleotide probe, served as controls for nonspecific binding of avidin.

Flow cytometric analyses. Flow cytometric analyses were performed with a Coulter EPICS-V flow cytometer (Coulter Electronics Inc., Hialeah, Fla.) equipped with a single 6-W argon ion laser. The 488-nm laser line was used for excitation with 250 mW of power focused to a spot (16.5 by 131 μm). Cells were passed through a 150-μm-diameter orifice, and FITC fluorescence was measured at wavelengths of 515 to 590 nm. The signals were collected over a 3-decade log scale using log amplifiers and normalized to 2.02-μm-diameter calibration beads (Polysciences, Inc., Warrington, Pa.). Data were collected in list mode (individual measurements of fluorescence per cell were collected and stored), and mean fluorescence values were compared and analyzed.

Photometer measurements. FITC fluorescence of cells hybridized with fluorescein and biotin probes was compared by measuring fluorescence on a Nikon P1 photometer system attached to a Zeiss IM 35 inverted epifluorescence microscope (FITC filter set). The system was interfaced with a computer and equipped with one shutter (Vincent Associates, Inc.) on the 50-W mercury light source and another on the photomultiplier tube. Individual cells of interest were located and aligned in the center of the viewing field, and the light path to the photometer was restricted by a filter with a 1.0-mm "pinhole," to admit light from the selected cell. The sample was illuminated for 800 ms. Total fluorescent light was converted to relative fluorescence units with commercially available software (Phoscan; Nikon). The mean relative fluorescence units ± 1 standard deviation was calculated from measurements of 20 randomly chosen cells for each treatment at a magnification of ×1,000.

Detection of mixed assemblages of protists from enrichment cultures and natural water samples. The ability of probes to label a wide range of naturally occurring protist cells was tested with mixed assemblages from enrichment cultures or natural water samples. To enrich for mixed populations of heterotrophic protists from seawater samples, yeast extract was added at a final concentration of 0.05% to 200 ml of seawater from the Sargasso Sea or Vineyard Sound (Woods Hole, Mass.). The cultures were kept in the dark at room temperature to selectively enrich for a mixed assemblage of heterotrophic protists. After 7 days, the cells were pelleted and fixed as described above. Samples were hybridized with EUK 1209R that was labeled with either fluorescein or biotin.

Natural samples of small protists from Eel Pond, Woods Hole, Mass., were collected by prefiltering 150 ml of seawater through 5- or 10-μm-pore-size Nitex screening. Samples were preserved with formaldehyde at a final concentration of 3.7% and stored at 4°C. After fixation for a minimum of 2 h, the samples were vacuum-filtered onto 1-μm-pore-size polycarbonate filters (Nuclepore) on a Millipore filtration apparatus until approximately 1 ml remained in the funnel. These crude concentrates of cells were transferred from the funnel to microcentrifuge tubes and centrifuged at 2,500 × *g* for 5 min. The supernatants were discarded, except for approximately 100 μl, which was used to resuspend the concentrated cells. Combinations of three or four biotin probes (EUK 309R, EUK 502R, EUK 1209R, and EUK B) were used for fluorescent detection of naturally occurring protists.

RESULTS

Flow cytometric analyses of protistan growth cycle and hybridization signal. Because the rRNA content of some microorganisms is known to be proportional to their growth rate (26), we expected that the physiological condition of protists would affect the amount of probe bound and therefore the hybridization signal (14). This effect was apparent in flow cytometric analyses of the ciliate used, an *Uronema* sp., sampled over its life cycle (Fig. 1A). The fluorescent signal from the ciliates, expressed as the cell/bead ratio, was highest in cells harvested during the early to mid-logarithmic phase of growth (Fig. 1B). A distinct peak in this signal occurred during early logarithmic growth. During stationary growth, the fluorescence signal due to probe binding was only slightly above background fluorescence resulting from nonspecific probe binding or autofluorescence.

Amplification of hybridization signal. On the basis of the results of the flow cytometric analyses, we attempted to amplify the fluorescence signal of probed cells by using biotin-labeled probes with fluorescein-labeled avidin and multiple probes. The rationale behind these attempts was to ensure that cells would be detectable in natural samples regardless of their physiological condition. The fluorescent intensities in these and subsequent hybridization experiments were based on average relative fluorescence units. These values were obtained by subtracting the control values from the experimental values in order to discount autofluorescence and nonspecific binding of the probes and fluorescein-labeled avidin.

The fluorescence of protists hybridized with the biotin-avidin system was significantly greater than that of cells labeled with oligonucleotide probes covalently linked to a single fluorescein moiety (three-way analysis of variance [ANOVA], $P < 0.01$; stippled versus white columns in Fig. 2). This result was true for cells harvested in the logarithmic and stationary growth phases. The fluorescent signal with the biotin-avidin system was two- to fivefold higher than the signal from the single fluorescein moiety over all the treatments tested. Fluorescence resulting from the binding of one biotin-labeled probe was also greater than that obtained from simultaneous labeling with three fluorescein-labeled probes targeting different sites in the eukaryotic small-subunit rRNA. The flagellate *Cafeteria* sp., however, displayed a high background fluorescence due to the binding of fluor-labeled avidin, which was localized in the food vacuoles of this protist.

The use of a combination of probes complementary to different target sites in the small-subunit rRNA was expected to additively increase the fluorescent signal proportionally to the number of probes added. The use of either two (EUK 309R and EUK 502R) or three (EUK 309R, EUK 502R and EUK 1209R) fluorescein-labeled probes or biotin-labeled probes versus a single probe (EUK 309R), however, did not significantly enhance the fluorescence of the ciliates (three-way ANOVA, $P = 0.07$) or flagellates (three-way ANOVA,

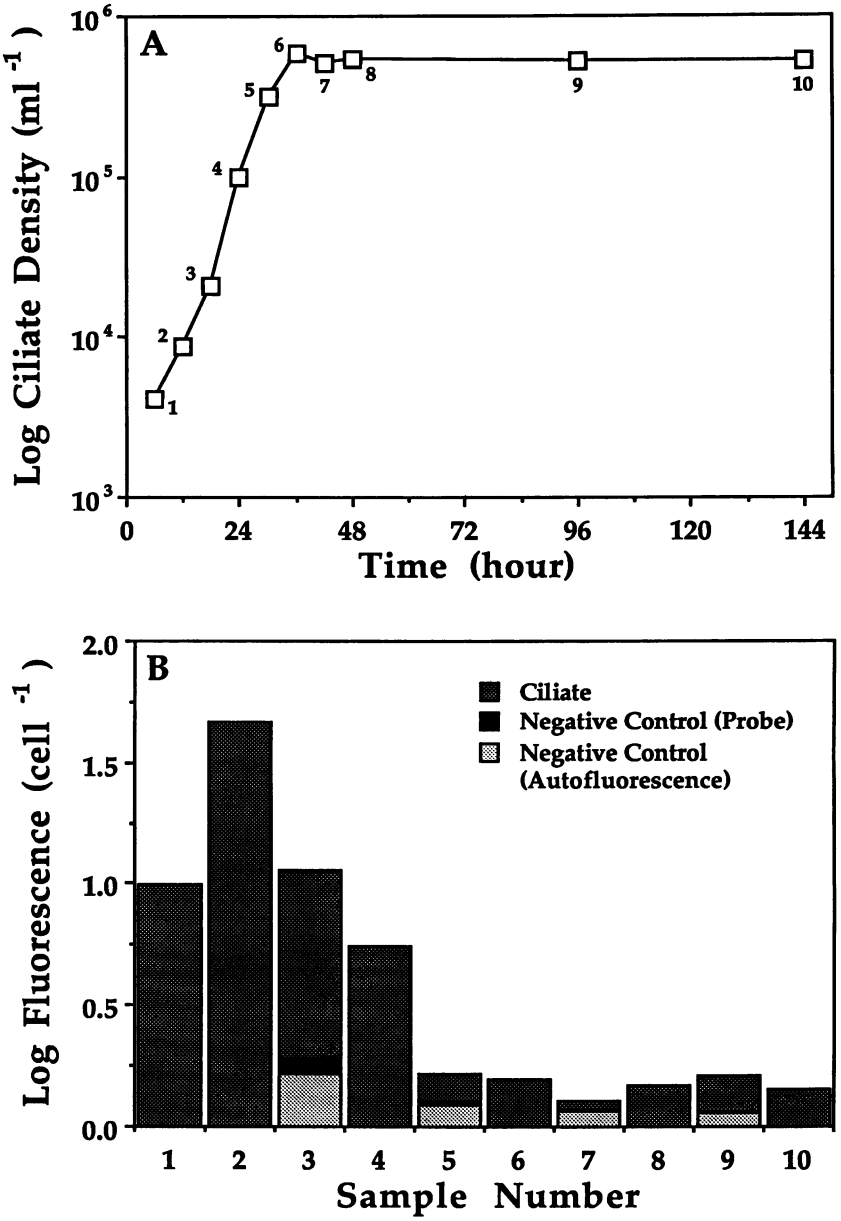


FIG. 1. (A) Growth curve of the ciliate *Uronema* sp. (B) Flow cytometric analysis of the ciliate hybridized with the fluorescein-labeled EUK 1209R probe. Fluorescence per cell was normalized to calibration beads. The sample numbers in panel B correspond with the sample numbers next to the datum points on the growth curve in panel A.

$P = 0.04$) at the $\alpha = 0.01$ significance level (Fig. 2). The lack of a significant difference among these treatments was due to the large standard deviations associated with the mean fluorescence values (10 to 30% of the means). Despite this high variability, there was a trend of increasing fluorescence with the use of multiple probes in all cases except the flagellates sampled during the stationary growth phase and hybridized with fluorescein-labeled oligonucleotide probes (Fig. 2d). The fluorescent signal in this latter treatment was only slightly more than that of negative controls, regardless of the number of fluorescein-labeled probes used.

Effect of growth phase on hybridization signal (photometer analyses). The fluorescent intensity due to probe binding in ciliates and flagellates was significantly greater when the cultures were in the logarithmic growth phase ($P < 0.01$). The fluorescence of ciliates in logarithmic growth (Fig. 2a) was approximately twofold greater than that of cells in stationary phase (Fig. 2b). The flagellates showed approximately a threefold increase in fluorescent intensity between cells in logarithmic and stationary growth phases (Fig. 2c and d, respectively). These results agreed closely with the flow cytometric data (Fig. 1), when samples taken from corresponding time points were compared. The fluorescence (cell/bead ratio) of the 24-h sample (sample 4 in Fig. 1) taken at mid-logarithmic growth phase was approximately three times greater than the 6-day sample (sample 10 in Fig. 1)

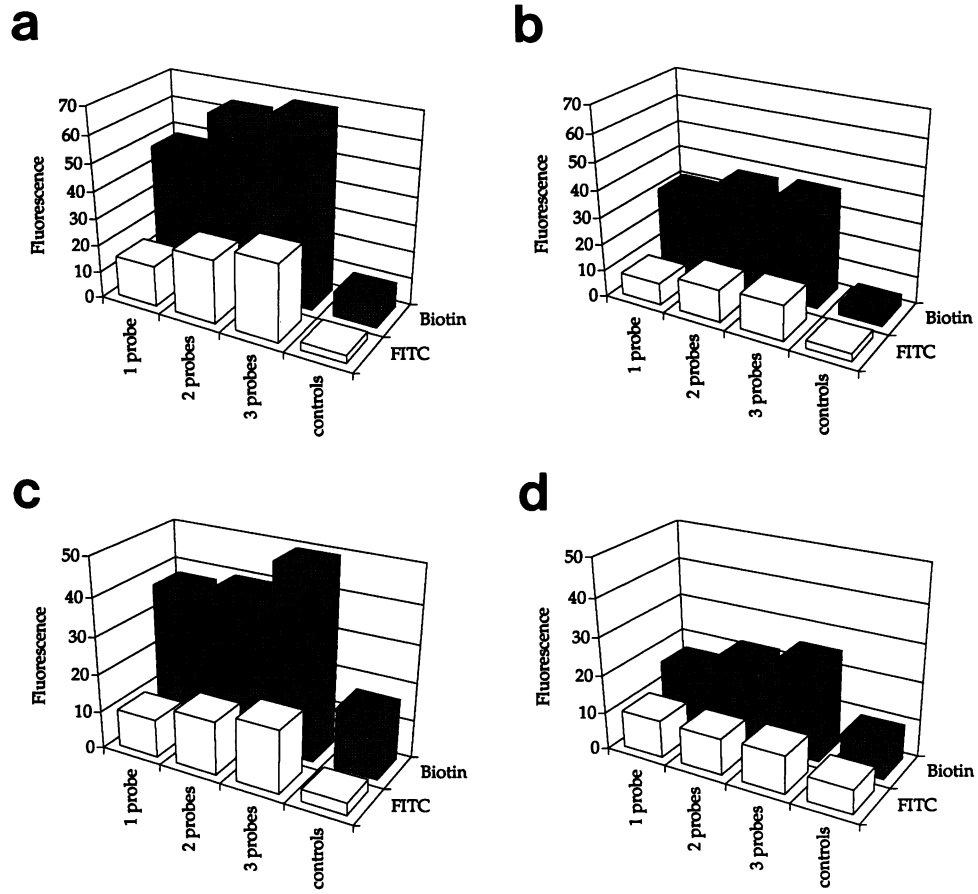


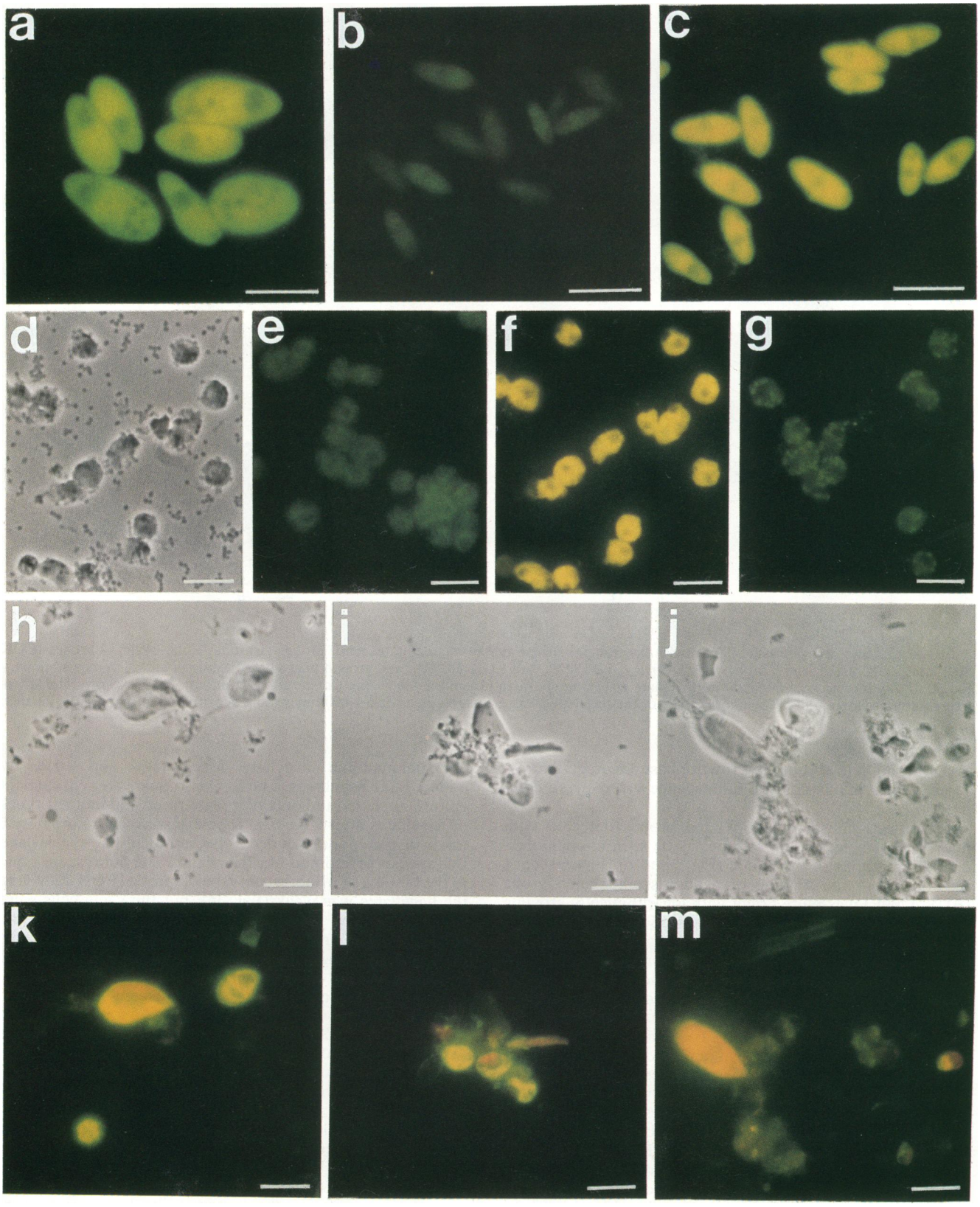
FIG. 2. Effects of probe label (biotin with fluorescein-labeled avidin versus fluorescein), probes (1 probe [EUK 309R], 2 probes [EUK 309R and EUK 502R], and 3 probes [EUK 309R, EUK 502R, and EUK 1209R]), and growth phase (logarithmic [a and c] versus stationary [b and d]) on fluorescence intensity in the ciliate *Uronema* sp. (a and b) and the flagellate *Cafeteria* sp. (c and d). Fluorescence intensity is expressed in relative units. The fluorescence of the flagellates was measured on a scale 1,000 times more sensitive than that for the ciliates.

taken during late stationary growth phase (average fluorescence values minus control fluorescence values were compared).

The effectiveness of biotinylated probes detected with fluorescein-labeled avidin can clearly be seen in epifluorescent micrographs of the ciliate (Fig. 3a to c). Fluorescence resulting from the binding of a fluorescein-labeled probe (EUK 1209R) was quite bright with logarithmically growing ciliates (Fig. 3a), whereas ciliates in stationary growth were barely visible (Fig. 3b). In contrast, ciliates from the same sample in the stationary growth phase were intensely stained by the same probe (EUK 1209R), when the probe was biotinylated and detected with fluorescein-labeled avidin (Fig. 3c).

Examination of natural, mixed assemblages of protists. Experiments using fluorescein- and biotin-labeled probes were repeated with mixed assemblages of protists from enrichments of seawater samples, as well as untreated, natural seawater samples. The goal of these experiments was simply to test the abilities of these probes to label a wide variety of naturally occurring protists. Enrichment of seawater with yeast extract initially promoted the growth of bacteria in the water sample. After approximately 2 days in continuous darkness, a bacterivorous ciliate dominated the culture. This domination by a ciliate was followed by a chrysomonad-like flagellate assemblage and then by bodonid-like flagellates. The Sargasso Sea water enrichment culture appeared to contain at least three codominant types of flagellates after 2 weeks. The cells were approximately 6 μm in diameter and included a chrysomonad-like species and two species of bodonid-like flagellates. The fluorescence due to the binding of a fluorescein-labeled probe (EUK 1209R) to the flagellates was weak (Fig. 3e), but the same cells were intensely stained with the same probe, biotinylated, and detected with fluorescein-labeled avidin (Fig. 3f). These cells were easily distinguished from the control which consisted of cells incubated with only fluorescein-labeled avidin (Fig. 3g). Because specific binding of fluorescein-labeled avidin consistently gave the highest background fluorescence compared with autofluorescence and negative-control probes (eubacteria and complements of the eukaryote probes), the fluorescein-labeled avidin controls are the only controls presented in our results.

Protozoa from enrichment cultures of Vineyard Sound seawater were also tested with fluorescein- and biotin-labeled oligonucleotide probes, as described above. Results of hybridizations were similar to the Sargasso Sea water enrichment cultures, except that the flagellate assemblage was dominated by tiny chrysomonad-like flagellates approximately 3 μm in diameter. These minute protists were readily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visualized with the biotin-labeled probes and fluorescein-labeled avidin (not shown).

Natural seawater samples were concentrated approximately 300 times in order to accommodate the small volume of the in situ hybridization samples. The mixed assemblage of protists was initially hybridized with a single, biotin-labeled probe, but the fluorescence of the protistan assemblage was weak. Experiments were repeated with a combination of three or four biotin-labeled probes (EUK 309R, EUK 502R, EUK 1209R, and EUK B), which significantly enhanced the fluorescent signal. A variety of protists from several different taxonomic groups were labeled by the biotin probes and fluorescein-labeled avidin (Fig. 3h to m). These cells were easily distinguished from background fluorescence, which was mainly associated with particulate detrital material. Cells associated with particulate material that were not apparent under transmitted light were also readily visualized with the biotin-labeled probes (Fig. 3l). Chlorophyll autofluorescence could still be detected in some of the flagellates as well (Fig. 3l to m).

DISCUSSION

Planktonic protists are a ubiquitous and taxonomically diverse group of aquatic microorganisms. The identification of these species in natural samples is often difficult because of their small size and the lack of distinctive morphological features that are easily distinguishable with a light microscope. Rapid, sensitive methods for identifying and enumerating these species are needed to facilitate studies of their population dynamics, community diversity, and biogeography. In this study, we tested the suitability of rRNA-based oligonucleotide probes in detecting several protist species. The use of fluorescein- or biotin-labeled eukaryote-specific probes allowed detection of a variety of protists in preserved seawater samples by epifluorescence microscopy. Our results demonstrate the feasibility of employing oligonucleotide probes for identifying and enumerating protist species, coupled with fluorescence methods for detecting the probe-binding cells.

A major obstacle in the application of oligonucleotide probes to natural samples of microorganisms has been the intensity of the probe signal in these cells. Increased sensitivity is necessary for labeling all target cells, because their rRNA content (and hence fluorescence due to probe binding) may vary widely as a function of their physiological state (14). In addition, sensitive methods of detection are required to maximize hybridization signal relative to background signal, which can be caused by nonspecific binding of probes or fluorochromes, or natural autofluorescence. Although other nonradioactive detection systems have been tested with bacteria, such as digoxigenin-labeled oligonucleotides detected with fluorescent anti-digoxigenin antibodies, these systems failed to increase the fluorescent signal resulting from probe binding (34). Enzymatically labeled probes have also recently been used for in situ hybridization and identification of prokaryotic cells (4). These probes are potentially more sensitive than fluorescently labeled probes, but pretreatment with enzymes or detergent may be necessary to improve the permeability of the cells. These pretreatments may prove to be too harsh for some delicate nanoplanktonic species.

Attempts at using other methods of signal amplification have produced variable results. Prior to our application of a biotin-avidin detection system, we tested oligonucleotides labeled with multiple fluors and polythymidine-tailed probes detected with short, fluor-labeled polyadenine oligonucleotides. Oligonucleotides labeled with multiple fluors were inefficient in hybridization reactions, mainly because of nonspecific binding to cell surfaces and slides. Polyadenine-tailed oligonucleotides were successfully applied, but these oligonucleotides appeared to be differentially permeative to different cell types.

The hybridization procedure with biotin probes and fluorescein-avidin described here is sensitive to cells in different physiological states and reasonably quick and requires minimal sample manipulation. Treatment with proteinase K or lysozyme was not necessary for the fluorescein-labeled avidin to penetrate freshly fixed cells. The interior of labeled protist cells was intensely fluorescent, except for the nucleus, which appeared as a dim circle within the cytoplasm. Unlabeled nuclei were good indicators of the probe specificity for cytoplasmic rRNA. The food vacuoles of cultured protists were often clearly unlabeled as well (Fig. 3a), but control cells incubated with fluorescein-labeled avidin and no oligonucleotide often contained fluorescent food vacuoles (Fig. 3g). The fluorescence of these controls results from the retention of fluorescein-labeled avidin in food vacuoles, which was probably due to the presence of endogenous biotin obtained from ingested bacteria. We have routinely observed bacteria in the food vacuoles of protozoa fluorescing brightly when fluorescein-labeled eubacterial probes were used as control probes for nonspecific binding to the protozoa. Nevertheless, the fluorescence from these fluorescein-labeled avidin controls was much weaker than fluorescence in the probed cells (Fig. 3f). It may be possible to reduce this background with the use of unlabeled avidin as a blocking reagent prior to hybridization.

In this study, we demonstrated the fluorescent in situ hybridization of natural assemblages of protists using biotinylated probes and fluorescein-labeled avidin. Further refinements of in situ hybridization will allow the procedure to be applied to natural samples filtered onto polycarbonate filters. In a recent study, bacteria growing in highly eutrophic artificial ponds were simultaneously hybridized with rRNA-targeted oligonucleotide probes and stained with the DNA-binding fluorochrome DAPI (20). Although the cells examined in these artificial, eutrophic ponds were likely

FIG. 3. Ciliates in mid-exponential (a) and late stationary (b) growth phase hybridized with the fluorescein-labeled EUK 1209R probe. The same sample of ciliate as in panel b hybridized with the biotin-labeled EUK 1209R probe and fluorescein-labeled avidin (c). Bars in panels a to c = 30 μm. Phase-contrast micrograph of a mixed population of heterotrophic flagellates from Sargasso Sea water enrichment cultures (d), and epifluorescence micrographs of the flagellates hybridized with the fluorescein-labeled EUK 1209R probe (e), biotin-labeled EUK 1209R probe (f), and controls hybridized with fluorescein-labeled avidin only (g). Bars in panels d to g = 10 μm. Protists from filtrates (5- and 10-μm-pore-size filters) of natural seawater samples collected from Vineyard Sound, Woods Hole, Mass. (h to m). Phase-contrast micrographs of protists hybridized with a combination of three biotin-labeled eukaryote probes (h) and with a mixture of four biotin-labeled eukaryote probes and fluorescein-labeled avidin (i and j). Corresponding epifluorescence micrographs (k to m) of the protists in panels h, i, and j. Bars in panels h to m = 10 μm. All exposure times were kept constant at 30 (a to c), 120 (d to g), and 60 (h to m) s to facilitate direct comparison of the treatments.

growing rapidly and so contained relatively large amounts of rRNA, only about 60% of the DAPI-stained bacteria could be detected using eubacterium-specific rRNA probes (20). Further development of signal amplification methods, such as that described in this report, should help alleviate some of this problem.

Increasing the sensitivity of oligonucleotide probes by methods such as biotin labeling and fluorescein-avidin detection facilitates the application of oligonucleotide probes for identifying and quantifying protists in natural samples. Growing data bases of small- and large-subunit rRNA sequences should allow for the future development of species- or genus-specific probes to detect and identify natural assemblages of protists. The application of these probes in ecological studies will lead to a greater understanding of the abundances and distributions of different taxonomic groups of protista in natural ecosystems and the roles that the protists play in energy flow in aquatic ecosystems.

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REFERENCES

1. **Amann, R., N. Springer, W. Ludwig, H.-D. Görtz, and K.-H. Schleifer.** 1991. Identification in situ and phylogeny of uncultured bacterial endosymbionts. Nature (London) **351:**161–164.
2. **Amann, R. I., B. J. Binder, S. W. Olson, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919–1925.
3. **Amann, R. I., L. Krumholz, and D. A. Stahl.** 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. **172:**762–770.
4. **Amann, R. I., B. Zarda, D. A. Stahl, and K. Schleifer.** 1992. Identification of individual prokaryotic cells by using enzyme-labeled, rRNA-targeted oligonucleotide probes. Appl. Environ. Microbiol. **58:**3007–3011.
5. **Azam, F., T. Fenchel, J. G. Field, J. S. Gray, L. A. Meyer-Reil, and F. Thingstad.** 1983. The ecological role of water-column microbes in the sea. Mar. Ecol. Prog. Ser. **10:**257–263.
6. **Buongiorno-Nardielli, M., and F. Amaldi.** 1970. Autoradiographic detection of molecular hybrids between rRNA and DNA in tissue sections. Nature (London) **225:**946–948.
7. **Caron, D. A.** 1983. Technique for enumeration of heterotrophic and phototrophic nanoplankton, using epifluorescence microscopy, and comparison with other procedures. Appl. Environ. Microbiol. **46:**491–498.
8. **Caron, D. A.** 1991. Evolving role of protozoa in aquatic nutrient cycles, p. 387–415. *In* P. C. Reid, C. M. Turley, and P. H. Burkill (ed.), Protozoa and their role in marine processes, vol. 25. Springer-Verlag, Berlin.
9. **Caron, D. A., and J. C. Goldman.** 1990. Protozoan nutrient regeneration, p. 283–306. *In* G. M. Capriulo (ed.), Ecology of marine protozoa. Oxford University Press, New York.
10. **Caron, D. A., E. L. Lim, G. Miceli, J. B. Waterbury, and F. W. Valois.** 1991. Grazing and utilization of chroococcoid cyanobacteria and heterotrophic bacteria by protozoa in laboratory cultures and a coastal plankton community. Mar. Ecol. Prog. Ser. **76:**205–212.
11. **Cox, K. H., D. V. DeLeon, L. M. Angerer, and R. C. Angerer.** 1984. Detection of mRNAs in sea urchin embryos by in situ hybridization using asymmetric RNA probes. Dev. Biol. **101:**485–502.
12. **Davis, P. G., and J. M. Sieburth.** 1982. Differentiation of phototrophic and heterotrophic nanoplankton in marine waters using epifluorescence microscopy. Ann. Inst. Oceanogr. **58**(Suppl.)**:**249–260.
13. **DeLong, E. F., and J. Shah.** 1990. Fluorescent, ribosomal RNA probes for clinical application: a research review. Diagn. Clin. Testing **28:**41–44.
14. **DeLong, E. F., F. S. Wickham, and N. R. Pace.** 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science **243:**1360–1363.
15. **Distel, D. L., E. F. DeLong, and J. B. Waterbury.** 1991. Phylogenetic characterization and in situ localization of the bacterial symbiont of shipworms (Teredinidae: Bivalvia) by using 16S rRNA sequence analysis and oligodeoxynucleotide probe hybridization. Appl. Environ. Microbiol. **57:**2376–2382.
16. **Gall, J. G., and M. L. Pardue.** 1969. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. Proc. Natl. Acad. Sci. USA **63:**378–383.
17. **Giovannoni, S. J., E. F. DeLong, G. J. Olsen, and N. R. Pace.** 1988. Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J. Bacteriol. **170:**720–726.
18. **Haas, L. W.** 1982. Improved epifluorescence microscopy for observing planktonic micro-organisms. Ann. Inst. Oceanogr. **58**(Suppl.)**:**261–266.
19. **Hahn, D., M. J. C. Starrenburg, and A. D. L. Akkermans.** 1990. Oligonucleotide probes that hybridize with rRNA as a tool to study *Frankia* stains in root nodules. Appl. Environ. Microbiol. **56:**1342–1346.
20. **Hicks, R. E., R. I. Amann, and D. A. Stahl.** 1992. Dual staining of natural bacterioplankton with 4′,6-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. Appl. Environ. Microbiol. **58:**2158–2163.
21. **Jurtshuk, R. J., M. Blick, J. Bresser, G. E. Fox, and P. Jurtshuk, Jr.** 1992. Rapid in situ hybridization technique using 16S rRNA segments for detecting and differentiating the closely related gram-positive organisms *Bacillus polymyxa* and *Bacillus macerans*. Appl. Environ. Microbiol. **58:**2571–2578.
22. **Lichter, P., C.-J. Chang Tang, K. Call, G. Hermanson, G. A. Evans, D. Housman, and D. C. Ward.** 1990. High-resolution mapping of human chromosome 11 by in situ hybridization with cosmid clones. Science **247:**64–69.
23. **Lovell, C. R., and Y. Hui.** 1991. Design and testing of a functional group-specific DNA probe for the study of natural populations of acetogenic bacteria. Appl. Environ. Microbiol. **57:**2602–2609.
24. **Malone, T. C.** 1971. The relative importance of nanoplankton and netplankton as primary producers in tropical oceanic and neritic phytoplankton communities. Limnol. Oceanogr. **16:**633–639.
25. **Medlin, L., H. J. Elwood, S. Stickel, and M. L. Sogin.** 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene **71:**491–499.
26. **Neidhardt, F. C., and B. Magasanik.** 1960. Studies on the role of ribonucleic acid in the growth of bacteria. Biochim. Biophys. Acta **42:**99–116.
27. **Sherr, B., and E. Sherr.** 1983. Enumeration of heterotrophic microprotozoa by epifluorescence microscopy. Estuar. Coastal Shelf Sci. **16:**1–17.
28. **Sherr, B. F., E. B. Sherr, and R. D. Fallon.** 1987. Use of monodispersed, fluorescently labeled bacteria to estimate in situ protozoan bacterivory. Appl. Environ. Microbiol. **53:**958–965.
29. **Sieburth, J. M.** 1984. Protozoan bacterivory in pelagic marine waters, p. 405–444. *In* J. E. Hobbie and P. J. I. Williams (ed.), Heterotrophic activity in the sea. Plenum Press, New York.
30. **Sogin, M. L., and J. H. Gunderson.** 1987. Structural diversity of eukaryotic small subunit ribosomal RNAs. Ann. N.Y. Acad.

Sci. **503**:125–139.

31. **Stahl, D., B. Flesher, H. R. Mansfield, and L. Montgomery.** 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Appl. Environ. Microbiol. **54**:1079–1084.
32. **Stahl, D. A., and R. Amann.** 1991. Development and application of nucleic acid probes, p. 205–248. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons Ltd., New York.
33. **Tsien, H. C., B. J. Bratina, K. Tsuji, and R. S. Hanson.** 1990. Use of oligodeoxynucleotide signature probes for identification of physiological groups of methylotrophic bacteria. Appl. Environ. Microbiol. **56**:2858–2865.
34. **Zarda, B., R. Amann, G. Wallner, and K.-H. Schleifer.** 1991. Identification of single bacterial cells using digoxigenin-labelled, rRNA-targeted oligonucleotides. J. Gen. Microbiol. **137**:2823–2830.